JOURNAL OF VIROLOGY, Nov. 1990, p. 5295–5300
0022-538X/90/115295-06$02.00/0

Vol. 64, No. 11

Concatameric Replication of Epstein-Barr Virus: Structure of the Termini in Virus-Producer and Newly Transformed Cell Lines

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Received 14 March 1990/Accepted 31 July 1990

The linear form of Epstein-Barr virus (EBV) DNA has homologous direct tandem repeats of approximately 500 bp at each terminus (TR). After infection, EBV DNA circularizes via the TR to form the intracellular episomal DNA. To analyze the mechanism of the synthesis of linear DNA through possible replicative intermediates, the terminal fragments were identified in the total intracellular DNA and the covalently closed circular DNA from a productively infected cell line after induction of replication or after treatment with an inhibitor of viral DNA synthesis. These studies indicate that some of the fused terminal fragments detected in the total intracellular DNA are replication-dependent forms which are selectively excluded from the covalently closed circular fraction and are eliminated after treatment with acyclovir. The EBV terminal restriction enzyme fragments were identified in three producer cell lines, each with a characteristic number of TR in the intracellular episomal DNA. Identification of the termini in cell lines established with the three virus strains revealed that the newly transformed cell lines had a greater number of TR than did the template DNA in the producer cell line. The increase in the number of TR in progeny episomes indicates that linear DNA is produced from concatameric replicative intermediates rather than from amplified catenated circular intermediates.

In the nonreplicative phase of infection, intracellular Epstein-Barr virus (EBV) DNA exists as an covalently closed circular (CCC) episome (18, 21). After induction of replication, the episomal DNA seems to serve as template for generation of the linear, virion DNA, which is synthesized by a virus-specific DNA polymerase, in contrast to the episomal form (8, 10, 21). Treatment of virus-producer cell lines with phosphonoacetic acid or acyclovir (ACV), specific inhibitors of EBV polymerase, greatly reduces the abundance of viral DNA; however, a small portion of the viral DNA in cell lines is resistant to inhibition by PAA and ACV and consists of CCC DNA (5, 6, 8, 29, 33). The inhibitors of the viral DNA polymerase also do not decrease the abundance of EBV DNA in cell lines that do not produce virus and that contain only episomal DNA, indicating that EBV episomal DNA is maintained by the host cellular DNA polymerase (5, 18). An origin of replication that confers the ability to be maintained episomally in the presence of an EBV gene product, EBNA1, has been identified (35).

Productive replication of the EBV genome may result from amplification of the episome, generating catenated, linked circles that are subsequently cleaved into the linear form. Alternatively, replication may occur through a rolling-circle or concatenated replicative intermediate (RI). A lytic origin of replication (ori_{lyt}) has been identified in the EBV genome (12). In permissively infected cell lines, plasmids containing these sequences are amplified in concatenated form after induction of EBV replication, suggesting that EBV may also be replicated via a concatenated RI. Pulse-field electrophoretic analyses of permissively infected cells revealed higher-molecular-weight forms that could reflect concatenated RI or topologically linked amplified circular DNA (J. Kolman and G. Miller, Abstr. Inc. Symp. EBV Assoc. Malig. Dis., p. 8, 1988).

EBV DNA has homologous direct tandem repeats of approximately 500 bp at each terminus (TR) (1, 7, 9, 17). In preparations of virion DNA, the terminal restriction endonuclease fragments are heterogeneous in size, varying by increments of 500 bp (9, 17). After infection, the linear termini join to form covalently closed episomal DNA. Variability in the number of TR at each linear terminus of EBV virion DNA generates fused termini after circularization that also contain variable numbers of TR (4, 7, 17, 22, 23). Analysis of the terminal restriction enzyme fragments of EBV revealed that in monoclonal cell lines and EBV-infected malignancies, the EBV DNA was clonal, with a homogeneous number of TR in the fused terminal restriction enzyme fragment representing the episomal DNA (3, 4, 23). The clonality of EBV genomes indicated, by extension, cellular monoclonality (23). In addition, the structure of the EBV termini provided evidence for viral replication, as indicated by the appearance of ladder arrays of terminal fragments representing linear forms of EBV DNA (14, 23). Evidence of linear arrays have also been detected in vivo in many lymphoproliferations (14).

In this study, to distinguish terminal fragments representing the template DNA, potential RI, and progeny linear DNA, the terminal fragments were identified in total intracellular DNA and in CCC DNA from virus-producer cell lines after induction of replication and after treatment with an inhibitor of viral replication. These analyses revealed that the CCC form contained a homogeneous number of TR and was detected as a single fused terminal restriction enzyme fragment. After induction of replication, fused terminal fragments were amplified in the total intracellular DNA but were not amplified in the CCC DNA fraction. These data indicate that some of the fused terminal fragments present in

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total intracellular DNA represent replication-dependent forms.

This analysis revealed that a given producer cell line has a constant number of TR in the template episomal DNA. Therefore, determination of the number of TR that are present in the linear progeny DNA arising from a specific template could clarify the form of the RI. If EBV DNA is amplified as a catenated circle, cleavage into the linear form within one or more TR would generate linear forms with variable numbers of TR at each terminus. Infection of new cells with this virus would generate episomal DNAs with fewer or the same number of TRs as are in the template DNA. In contrast, if the episome is amplified via a concatenated RI, then random cleavage within the TR could generate after infection progeny episomes that contain all possible numbers of TR, ranging from a few to almost twice that in the parental template. To assess the total number of TR in linear progeny, the fused terminal fragments were identified in cell lines established by infection in vitro with virus produced from three cell lines with a known, constant number of TR in the episomal DNA. These studies revealed that in each case, progeny molecules that contained the full possible range of copies of TR were synthesized. The range in variation in the number of TR was proportional to the number of TR in the template DNA, with the maximum number of TR in the progeny molecules approaching twice that in the parental template. The generation of progeny molecules with increased numbers of TR suggests that EBV replicates as a concatenated RI.

MATERIALS AND METHODS

Cell lines, virus purification, and infection in vitro. The B95-8 and AG876 lymphoid cell lines were maintained in RPMI–10% fetal calf serum. The NPC-KT, and A2L/AH cell lines were maintained in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. B95-8 and AG876 are partially permissive cell lines that were induced to greater permissivity by treatment for 7 days with 20 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) per ml (Sigma Chemical Co., St. Louis, Mo.) (19, 36). The EBV-positive NPC-KT cell line is an epithelial hybrid cell line established by fusion of a primary nasopharyngeal carcinoma tissue with an adenoidal epithelial cell line, AD/AH (30, 31). A2L/AH is an epithelial hybrid cell line produced by fusion of AD/AH with a lymphoblastoid cell line established by infection with B95-8 virus (32). Virus production was induced in the NPC-KT and A2L/AH cell lines by treatment with 60 μg of 5-iodo-2″-deoxyuridine (IUdR) per ml for 3 days, followed by 3 days of culture without IUdR.

Virus replication was inhibited in the producer cell lines by treatment with 100 μM ACV (5, 6). Intracellular DNA was extracted from total cellular lysates or CCC DNA prepared after alkaline extraction (11, 23).

Lymphocytes were purified from neonatal umbilical cord blood or from adult peripheral blood after centrifugation through a Ficoll step gradient. The nonadherent mononuclear cells were infected with supernatant from the induced NPC-KT, AG876, or A2L/AH cell line and cultured for 3 to 4 weeks in vitro. Peripheral blood lymphocytes were cultured in the presence of 2 ng of cyclosporin A per ml (34).

Identification of the EBV termini. EBV intracellular or virion DNAs were digested with *Bam*HI, subjected to electrophoresis through a 0.6% agarose gel, and transferred to nitrocellulose. The right-terminal *Bam*HI fragments were identified by hybridization with a ^{32}P-labeled single-stranded RNA probe synthesized from a 1.9-kb *Xho*I fragment cloned into the pGEM2 vector (Promega, Madison, Wis.), using SP6 polymerase (23). This fragment represents unique DNA adjacent to the terminal repeats at the right end of the genome. The left-terminal restriction enzyme fragments were identified by using the *Eco*RI-*Bam*HI portion of the *Eco*RI I fragment, representing unique DNA adjacent to the terminal repeats at the left end of the genome, labeled with ^{32}P by nick translation (23, 24).

RESULTS

Identification of terminal fragments during replication. To identify and distinguish the terminal fragments representing the episomal DNA from terminal fragments in RI and progeny DNA, the terminal fragments of the B95-8 strain of EBV were identified. The right-terminal *Bam*HI fragment of B95-8 EBV contains approximately 3.5 kb of unique DNA and varying numbers of TR, whereas the left-terminal *Bam*HI terminus of EBV contains approximately 4 kb of unique DNA with multiple copies of TR (1, 7, 23). The fused terminal fragments, which contain 7.5 kb of unique DNA, can be identified by their larger size and will hybridize to probes representing unique DNA from either end of the genome (23).

Southern blots were prepared with total intracellular and CCC DNA digested with *Bam*HI from B95-8 cells that were untreated or treated with TPA, ACV, or TPA plus ACV for 7 days. The filters were hybridized to recombinant single-stranded RNA or DNA probes representing unique DNA sequences adjacent to the repeat sequences from the left or right end of the EBV genome (Fig. 1). As reported previously, multiple fused terminal fragments were identified with the probes from the left and right termini, which ranged in size from 8.9 to 10.4 kb (4, 23). A ladder of fragments representing linear termini was also detected in the total intracellular DNA from uninduced or TPA-treated B95-8 cells. After treatment with ACV, an inhibitor of the EBV-encoded DNA polymerase, most of these fragments were greatly reduced, with a predominant single 10.4-kb fused terminal fragment remaining. These results indicated that the multiple fused terminal fragments and the linear arrays of terminal fragments were synthesized by the viral DNA polymerase and were replication-dependent forms. The viral DNA, which was resistant to ACV treatment and has been previously shown to consist of CCC DNA, contained homogeneous viral DNA with a constant number of TR.

To confirm that the endogenous episomal DNA contained a constant number of TR, CCC DNA was selectively extracted. This DNA fraction also contained a single 10.4-kb fused terminal fragment whose abundance was largely unaffected by induction of replication with TPA or treatment with ACV (Fig. 1) (11). Consistently high background in the lane containing CCC DNA from TPA-induced cells was probably due to contaminating small, single-stranded DNA fragments from the nicked viral DNA, characteristic of herpes virion DNA (16). These results confirmed that in B95-8 cells, the CCC episomal DNA which is maintained by the cellular enzyme contains a homogeneous number of TR and that the abundance of the episomal DNA is unaffected by induction or inhibition of viral replication.

In contrast, in the total intracellular DNA, heterogeneous fused termini ranging in size from 8.9 to 10.4 kb were detected. These fragments increased after induction of replication and were greatly reduced by treatment with ACV. These additional fused terminal fragments therefore must be

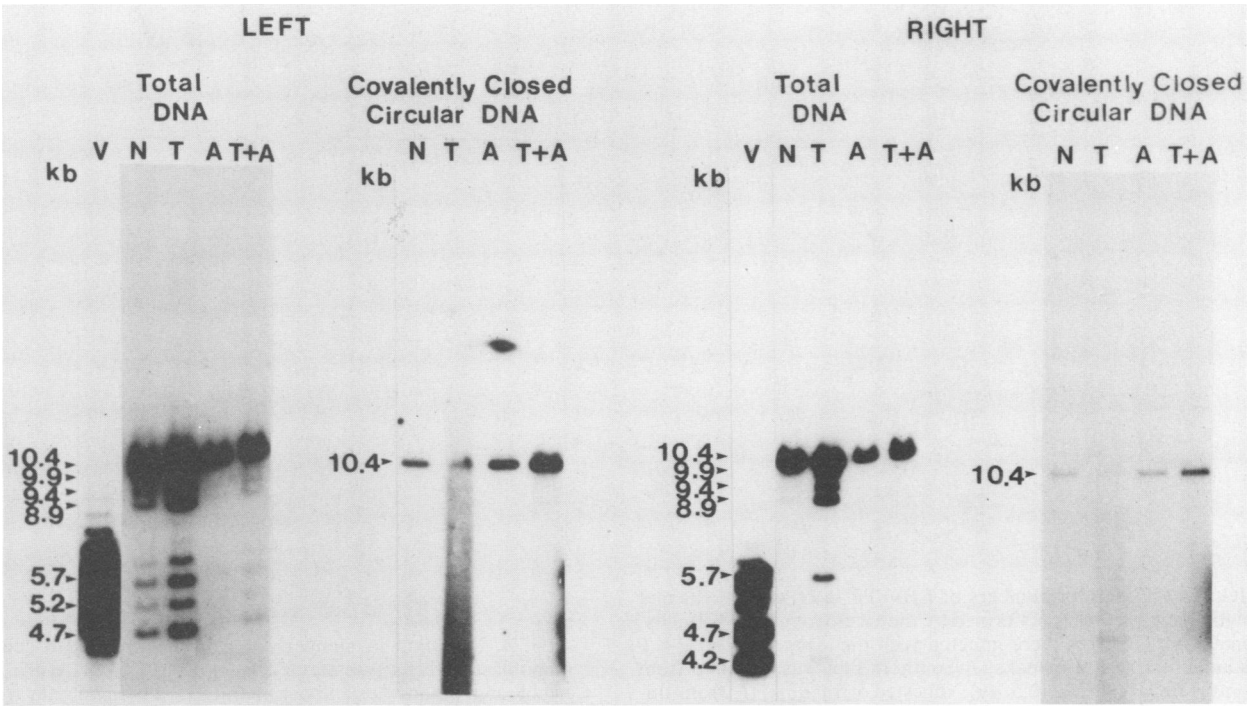

FIG. 1. Analysis of EBV termini in intracellular and CCC DNAs from B95-8 cells. B95-8 cells were untreated (N), induced by 20 ng of TPA per ml (T), treated with 100 μM ACV (A), or incubated with TPA plus ACV for 7 days (T + A). A Southern blot, prepared with intracellular DNA (5 μg) and CCC DNA from 2×10^6 cells digested with *Bam*HI, was hybridized to the ^{32}P-labeled RNA transcribed with SP6 polymerase from the *Xho*I 1.9-kb fragment representing unique DNA adjacent to the right-terminal repeats (RIGHT). After autoradiography, the nitrocellulose filter was treated with boiling water to remove the *Xho*I probe and rehybridized to an *Eco*RI-I probe labeled with ^{32}P by nick translation. This probe represents unique DNA adjacent to left-terminal repeats (LEFT).

synthesized by the viral DNA polymerase and represent replication-dependent forms. Fused terminal fragments that were smaller than the template DNA were also detected in the HR-1 cell line after induction with butyrate (14). These new forms with differing numbers of TR may represent RI formed from progeny DNA.

Analysis of template DNA and episomal DNA produced by infection with progeny virion DNA. These data indicated that the homogeneous episomal DNA is a template for the generation of replication-dependent forms which are amplified by the viral DNA polymerase. These forms included virion DNA represented by ladder arrays of terminal restriction enzyme fragments and increased fragments representing fused termini. Since each EBV-infected cell line has a characteristic number of TR, analysis of the total number of TR in the progeny DNA molecules should elucidate the form of the RI. Random cleavage within any of the copies of TR in a concatameric linear template containing four copies of TR would produce progeny molecules whose total number of TR could vary between two and six (Fig. 2). If the template DNA contained 8 copies of TR, the variation in the number of TR in the progeny molecules would increase and could vary from 2 to 14.

Analysis of the EBV terminal fragments after infection with EBV produced by the nasopharyngeal carcinoma hybrid cell line (NPC-KT) revealed that NPC-KT virus efficiently circularizes or forms concatamers (25–27). The restriction terminal fragment(s) of episomal DNA in NPC-KT cells was identified on Southern blots prepared with intracellular DNA digested with *Bam*HI from untreated NPC-KT cells and cells treated with IUdR to induce viral replication. Hybridization with the *Xho*I 1.9-kb probe representing the unique DNA from the right terminus identified a single 9.4-kb fragment in mock-treated NPC-KT cells which would contain four copies of TR (Fig. 3). In IUdR-induced cells, in addition to an amplified 9.4-kb fused fragment, a ladder of linear termini was identified. Hybridization with the *Eco*RI-I probe representing unique DNA from the left terminus also identified the single 9.4-kb fragment in mock-treated cells

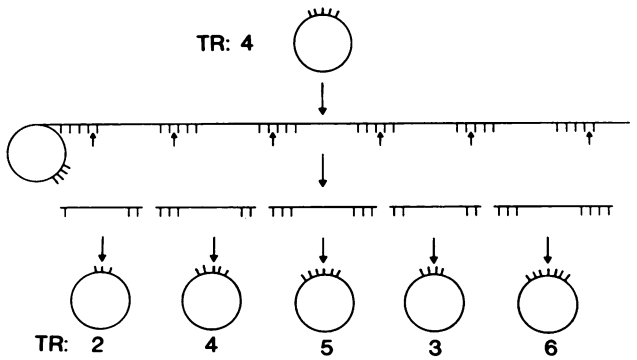

FIG. 2. Concatameric replication of EBV: variation of numbers of TR in episomal forms in virus-producer cells and in infected cells. The top line shows the synthesis and cleavage of concatemeric DNA; the middle line shows the progeny linear forms with variable numbers of TR; the bottom line shows the progeny circular forms that would be produced from a concatameric template containing four copies of TR.

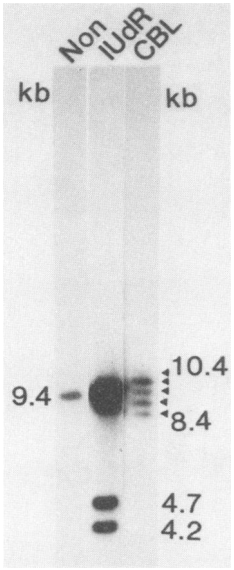

FIG. 3. Variation of numbers of EBV TR in NPC-KT cells and neonatal lymphocytes transformed by the virus from NPC-KT cells. Neonatal lymphocytes were infected with the virus from NPC-KT cells and cultured for 4 weeks. A Southern blot was prepared from the intracellular DNAs (2.5 μg), digested with *Bam*HI, from untreated cells (lane Non), from IUdR-induced NPC-KT cells (lane IUdR), and from cord blood lymphocytes transformed by the virus from NPC-KT cells (lane CBL). The blot was hybridized to the *Xho*I 1.9-kb probe for the EBV right-terminal fragment.

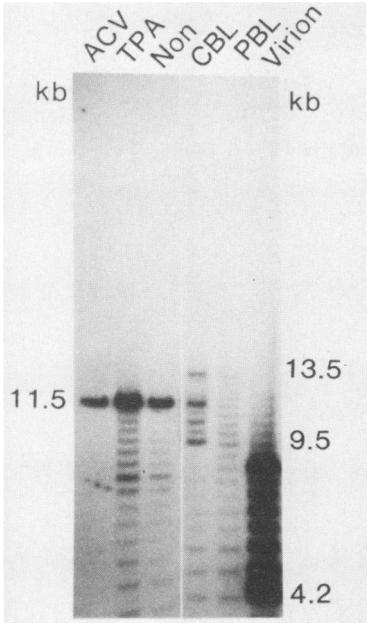

FIG. 4. Variation in numbers of EBV TR in AG876 cells and lymphoblastoid cells transformed by the virus from AG876 cells. Cord blood lymphocytes and peripheral blood lymphocytes from an EBV-seropositive person were infected with AG876 virus and were cultured for 3 weeks in the absence and presence, respectively, of cyclosporin A (2 ng/ml). Virion DNA (10 ng) (lane Virion) and intracellular DNAs (2.5 μg) from ACV-, TPA-, and untreated AG876 cells and from cord blood lymphocytes and peripheral blood lymphocytes transformed by virus from AG876 cells (lanes ACV, TPA, Non, CBL, and PBL, respectively) were digested with *Bam*HI. The Southern blot was hybridized with the *Xho*I 1.9-kb probe for the EBV right-terminal fragment.

and a ladder of linear forms and an amplified 9.4-kb fragment in IUdR-induced cells (data not shown). Amplification of the 9.4-kb fused fragment after induction of virus replication suggested that this fragment represents the intermediate for the synthesis of linear DNA.

The fused termini that were formed upon transformation of lymphoblastoid cells with NPC-KT virus were characterized. The fused terminal fragments produced by infection with NPC-KT virus were identified in cord blood lymphocytes transformed by the virus and ranged in size from 8.4 to 10.4 kb (Fig. 3). These fused terminal fragments would contain between two and six copies of the TR, identical to the number predicted by the model in Fig. 2.

The structure of termini was also characterized in the virus-producer lymphoid cell line AG876. A single fused 11.5-kb fragment containing eight copies of TR was identified in AG876 cells treated with ACV (Fig. 4). The single 11.5-kb fused fragment was amplified after induction of replication. The right-terminal fragments in the purified virion DNA preparations ranged in size from 4.2 to 11.5 kb, which would contain two to eight copies of TR. Mononuclear lymphoid cells purified from umbilical cord blood or adult peripheral blood lymphocytes were infected with AG876 virus. The EBV terminal fragments were identified in the total intracellular DNA 3 to 4 weeks after infection. The AG876 linear virion DNA circularized after infection of neonatal lymphocytes and adult peripheral blood lymphoblastoid cells to form predominant fused terminal fragments that varied in size from 9.5 to 13.5 kb, representing 4 to 12 copies of TR. A ladder of fragments representing the mixture of linear and fused termini ranging in size from 4.2 to 13.5 kb was also detected, indicating that the cell lines were partially permissive for EBV replication.

A2L/AH is an epithelial hybrid cell line of human adenoid epithelial cells and lymphoblastoid cells transformed by virus from B95-8 cells (32). In A2L/AH cells, a single 16.9-kb fused terminal *Bam*HI fragment, containing approximately 17 TR, and a ladder of linear termini were identified. The fused termini detected in lymphoid cell lines established by infection with A2L/AH virus ranged in size from size 8.9 to 22.4 kb (Fig. 5). Fused terminal *Bam*HI fragments of this size would contain approximately 3 to 30 copies of TR.

As described above, the RI template for NPC-KT virus DNA apparently contains a single joined terminal fragment of 9.4 kb representing four copies of TR (25). Upon entry into cells, NPC-KT virus circularized to form fused termini ranging in size from 8.4 to 10.4 kb, representing two to six copies of TR. Similarly, although linear virion DNA was synthesized in AG876 cells from a template containing 8 copies of TR, the virus circularized during transformation of lymphoblastoid cells to generate episomal forms with fused termini containing 4 to 12 copies of TR. The range of episomal DNA produced by infection with A2L/AH virus, produced from a template with 17 copies of TR, was more heterogeneous and varied from 3 to 30 copies of TR. In each case, the range of heterogeneity in TR in the cell lines produced with the progeny virus reflected the number of TR in the template DNA (n) and the number of TR in some of the progeny molecules increased to the near maximum (2n−2) predicted from a linear, concatenated RI.

DISCUSSION

It is difficult to characterize the RI of the large herpesvirus genomes (2, 13). The very large size of the genomes has

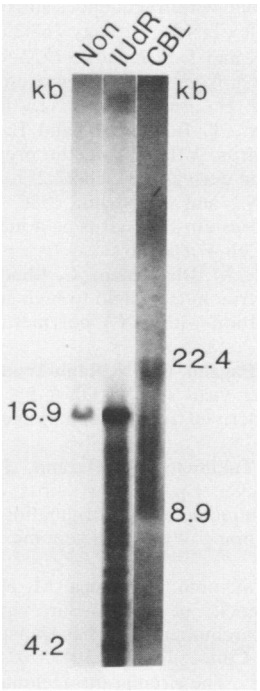

FIG. 5. Variation in numbers of EBV TR in A2L/AH cells and cord blood lymphocytes transformed by virus from A2L/AH cells. Cord blood lymphocytes were infected with virus from A2L/AH cells and cultured for 4 weeks. Intracellular DNAs (2.5 μg) from uninduced and IUdR-induced A2L/AH cells and from cord blood lymphocytes transformed by virus from A2L/AH cells (lanes Non, IUdR, and CBL, respectively) were digested with *Bam*HI. A Southern blot was prepared and hybridized with the *Xho*I 1.9-kb probe for the EBV right-terminal fragment.

complicated analysis by electron microscopy, which detected very large, tangled RI, without evidence of theta structures (13). Similarly, analysis of sedimentation rates or electrophoretic mobility reveals very large RI (2). During replication of herpes simplex virus, terminal restriction enzyme fragments cannot be identified (13). Failure to detect termini suggests that the RI is a concatenated linear form perhaps produced from a rolling circle. Alternatively, the RI might be catenated, topologically linked circles.

The mechanism of EBV DNA replication during the replicative cycle has also not been established. However, unlike herpes simplex virus, which has direct repeat elements at the termini and inverted repeat elements which generate multiple isomeric forms through recombination and inversion, EBV does not isomerize and the TR are found only at the termini (1, 7). This aspect of EBV DNA structure allows for the identification of terminal fragments throughout the replicative process. In the herpes simplex virus genome, at least one copy of the reiterated *a* sequence is found at each terminus in the mature progeny (20). Similarly, in EBV, the most frequent cleavages retain at least one TR at each end. The episomes that are formed with the progeny virus from such cleavages contain at least two TR, with a likely maximum of 2n−2.

There are two possible mechanisms for viral DNA replication from an episomal template. The circles can be nicked to form either a rolling circle or a concatenated RI, or the circle could directly replicate via a theta-shaped RI (15). Whichever mechanism is invoked, one would expect to see an increase in the abundance of the fused terminal restriction fragment during replication. Either form of RI would be expected to migrate as a very large molecule in a pulse-field analysis.

In the theta model for the RI, the resident plasmids are amplified by viral enzyme, and the newly synthesized catenated circular genomes are cleaved to a linear form (28). In this case, fused termini would represent circular DNA. In the rolling-circle model, the fused termini would represent DNA concatemers. Cleavage of both concatemers and circular intermediates, which may take place at different positions in the region of TR, generates a ladder of terminal restriction fragments in linear DNA which are heterogeneous in size by increments of 500 bp. However, linear DNA derived from concatemers would circularize to produce fused termini containing both greater and fewer numbers of TR. If the linear forms were derived from circular intermediates, circularization would form joint termini containing the same numbers of TR as are in the fused terminus of episomal DNA from which linear DNA is derived. If the circular intermediates were cleaved at more than one site in the region of TR, then circles formed after infection with the virus could contain fewer total numbers of TR. The data obtained in this study support the model that EBV DNA replication which is mediated by the viral DNA polymerase proceeds via linear concatemers as RI, perhaps by a rolling-circle mechanism.

Recently, the EBV origin of DNA replication that functions during lytic infection, termed ori_{lyt}, has been identified (12). Replication that is mediated through ori_{lyt} depends on the EBV DNA polymerase and yields a concatemeric molecule (12). The data presented here suggests that the intact viral genome also replicates via a concatenated RI. Moreover, the detection of non-CCC DNA which contains fused termini with fewer copy numbers of TR that are synthesized from a homogeneous episomal template suggests that progeny molecules may also form RI.

The terminal restriction enzyme fragments have been previously identified in the prototype EBV-producer cell line B95-8 (4, 23). These studies indicated that B95-8 contained at least four fused terminal fragments and linear molecules with heterogeneous termini (4, 23). The presence of multiple fused termini in B95-8 could reflect the proliferation of several EBV-infected clones or indicate that the original cell was infected by more than one virion, resulting in circularized intracellular EBV genomes with differing numbers of TR. However, analysis of the immunoglobulin gene rearrangement indicated that the B95-8 cell line was monoclonal (4). The data presented here indicate that the episomal DNA in the B95-8 cell line is also clonal, with a homogeneous number of TR, in agreement with the clonality indicated by immunoglobulin gene rearrangement.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant CA32979 from the National Cancer Institute and grant MV354 from the American Cancer Society.

We thank M. Seiki and J. S. Pagano for helpful discussions.

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